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Decreased Circulating Fas Ligand in Patients With Familial Combined Hyperlipidemia or Carotid Atherosclerosis

Normalization by Atorvastatin

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OBJECTIVES We sought to study whether patients with familial combined hyperlipidemia (FCH) or carotid atherosclerosis have modified circulating solubilized Fas ligand (sFasL) levels, as well as the potential modifications by atorvastatin. We also examined the effect of atorvastatin on FasL expression and sFasL release in cytokine-stimulated cultured human endothelial cells (ECs).

BACKGROUND In normal situations, FasL is expressed in most cells, including ECs. Proinflammatory stimuli can downregulate its expression in ECs and facilitate the vascular infiltration of inflammatory cells.

METHODS We have measured sFasL plasma levels (by ELISA) in 58 patients with FCH, 14 normocholesterolemic patients with carotid atherosclerosis, and 15 healthy volunteers. We analyzed FasL expression (by Western blot analysis) and sFasL release in cultured ECs stimulated with tumor necrosis factor (TNF)-alpha.

RESULTS Solubilized FasL levels were decreased in hyperlipidemic patients (49 pg/ml), as compared with healthy volunteers (123 pg/ml, $p < 0.0001$). Patients were randomized to atorvastatin ($n = 28$) or bezafibrate ($n = 30$) during 12 months. Atorvastatin treatment increased sFasL concentrations (111 pg/ml, $p < 0.0001$), reaching normal values. However, treatment with bezafibrate only marginally affected sFasL (85 pg/ml, $p < 0.05$). Solubilized FasL was also diminished in patients with carotid atherosclerosis (39 pg/ml), and intensive treatment with atorvastatin normalized sFasL levels (90 pg/ml, $p = 0.02$). Finally, atorvastatin prevented the diminution of FasL expression and sFasL release elicited by TNF-alpha in cultured ECs.

CONCLUSIONS Patients with FCH or carotid atherosclerosis have decreased circulating sFasL levels, probably indicating endothelial dysfunction, but treatment with atorvastatin restored normal blood levels. These data provide a novel effect of atorvastatin and add support for the well-known anti-inflammatory properties of statins. (J Am Coll Cardiol 2004;43:1188–94) © 2004 by the American College of Cardiology Foundation

The Fas-Fas ligand system is a representative pathway of apoptosis-signaling receptor molecules. The Fas ligand (FasL or CD95L) is a type II membrane protein that is homologous to tumor necrosis factor (TNF)-alpha (1). The Fas ligand induces apoptosis or programmed cell death when bound to its membrane receptor Fas (also known as apoptosis antigen-1 [APO-1] or CD95). Engagement of FasL promotes the trimerization of Fas and the formation of a signaling complex of molecules linked by protein–protein interactions with the cytoplasmic portion of the receptor. Although the expression of FasL was originally considered restricted to activated T lymphocytes and natural killer cells, it has also been identified in several organs and tissues (2). The membrane-bound FasL is converted to a solubilized form (sFasL) by a metalloproteinase (3). The conversion of membrane FasL to its solubilized form decreases its apoptotic activity, although sFasL retained its capacity to interact with Fas, and restoration of total cytotoxic activity has been achieved both in vitro and in vivo by the addition of cross-linking antibodies (4). Expression of FasL has been classically described to be induced during an immune-enhanced response (5,6). However, the constitutive expression of FasL has been linked to tissue-specific regulation of various immune processes. In this way, the expression of functional FasL by some tissues contributes to their immune-privileged status by impairing survival of infiltrating leukocytes (7). Also, recent reports have demonstrated that endothelial cells (ECs) express FasL constitutively and therefore regulate leukocyte vessel wall infiltration (8). Familial combined hyperlipidemia (FCH) is a common form of dyslipidemia characterized by increased low-density lipoprotein (LDL) cholesterol and triglyceride serum levels, usually associated with low concentrations of high-density lipoprotein (HDL) cholesterol (9). This lipid phenotype carries a high risk of premature coronary artery disease (10,11). Fibric acid derivatives and hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are frequently used in combined hyperlipidemia

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Abbreviations and Acronyms

EC	= endothelial cell
FCH	= familial combined hyperlipidemia
GGPP	= geranylgeranyl pyrophosphate
HDL	= high-density lipoprotein
HMEC-1	= human microvascular endothelial cell
HMG-CoA	= hydroxymethylglutaryl-coenzyme A
LDL	= low-density lipoprotein
PBST	= phosphate-buffered saline containing 0.1% Tween-20
sFasL	= solubilized Fas ligand
TNF	= tumor necrosis factor

(12). Large clinical trials have demonstrated that statins reduce the risk of myocardial infarction and stroke (13). Increasing evidence suggests that statins exert cardiovascular effects beyond the lowering of serum cholesterol levels (14).

Sustained hyperlipidemia is associated with subendothelial lipoprotein deposits in atherosclerosis-prone areas of the vasculature, triggering endothelial adhesion molecule expression and monocyte infiltration, probably in an attempt to remove the lipid deposits. We hypothesized that if FasL expression in normal ECs regulates leukocyte infiltration, high cholesterol levels would be associated with a decrease in FasL expression, and therefore sFasL would vary accordingly. We first studied sFasL levels in patients with FCH at baseline and 12 months after randomization to treatment with atorvastatin or bezafibrate, and we then compared them to a matched control population. We also studied sFasL levels in patients with clinical atherosclerosis without marked hyperlipidemia and evaluated the effect of short-term, high-dose statin treatment on sFasL. Finally, we assessed the in vitro effect of atorvastatin on FasL expression and sFasL release in cytokine-stimulated cultured human ECs.

METHODS

Patients with FCH. The hyperlipidemic population included 58 patients participating in the Atorvastatin versus Bezafibrato Mixed Hyperlipidemia (ATOMIX) trial, a one-year randomized, double-blinded, multicenter study of adult patients with combined hyperlipidemia in which atorvastatin and bezafibrate therapies were compared (15). The characteristics of the study population are shown in Table 1.

Patients were included in the trial if, after discontinuation of any lipid-regulating drug, formal dietary counseling and good compliance with the prescribed diet, and a six-week run-in period of placebo treatment, they had a mean (of 2 consecutive analyses at weeks −4 and −2) triglyceride level of <500 and ≥200 mg/dl, respectively, in addition to LDL cholesterol <250 and >190, 180, 160, or 135 mg/dl, depending on the global risk status (low, moderate, high, or presence of coronary heart disease, respectively), according to the European Atherosclerosis Society (EAS) recommendations (16). Patients were ineligible if they were pregnant or nursing or had dysbetalipoproteinemia, chronic liver disease or hepatic dysfunction, nephrotic syndrome or renal insufficiency, a body mass index >30 kg/m^2, known hypersensitivity to HMG-CoA reductase inhibitors or fibrates, and a weekly average consumption of more than 130 g of alcohol. Patients with uncontrolled hypertension, hypothyroidism or uncontrolled type II diabetes mellitus were also excluded. Patients must not have had a myocardial infarction, angioplasty, severe or unstable angina pectoris, or any other cardiovascular event resulting in hospitalization during the six months preceding the study. Drugs known to affect lipid levels or to interact with study medication were not allowed. Dietary compliance was assessed at baseline, and noncompliant patients were also excluded.

Patients were randomly assigned to receive a daily dose of either 10 mg atorvastatin or 400 mg bezafibrate. After 8 and 16 weeks of double-blinded treatment, the dose of atorvastatin could be doubled, according to the modified EAS LDL cholesterol target guidelines (LDL cholesterol ≤175, 155, 135, or 100 mg/dl for patients at low risk, moderate risk, high risk, or with coronary heart disease, respectively) (16). At week 26, if after two consecutive titrations, LDL cholesterol levels were still above target, open-label colestipol (3 sachets of 5 g/day) was recommended for the rest of the study in both arms. The lipid values of randomized patients were kept unknown to both the patient and the investigator until the end of the study.

The control population included 15 healthy adults matched by age and gender.

Patients with atherosclerotic carotid disease. Fourteen normocholesterolemic patients with carotid atherosclerosis (carotid stenosis ≥70%, as diagnosed by Doppler echocardiography) and without previous statin therapy were randomized to receive 80 mg/day atorvastatin (n = 7) or usual care (n = 7) during four to six weeks before they underwent scheduled carotid endarterectomy at the Hospital Clínico San Carlos, Madrid, Spain (Prof. J. Serrano). Written, informed consent was obtained from all patients before enrollment. The study was approved by the Hospital's Ethical Committee, in accordance with the institutional guidelines.

Patients were excluded from the trial if they were pregnant or nursing, had some inflammatory disease or tumor, or had been treated with hypolipemic or anti-inflammatory

Table 1. Characteristics of Hyperlipidemic Patients

Variable	Atorvastatin Group (n = 28)	Bezafibrate Group (n = 30)	p Value
Age (yrs)	49.9 ± 11	52.9 ± 11	0.29
Body mass index (kg/m^2)	27.7 ± 2	27.4 ± 2	0.56
Gender			0.25
Men	24 (86%)	22 (73%)	
Women	4 (14%)	8 (27%)	
Diabetic	2 (7%)	4 (13%)	0.44
Smoker	9 (32%)	10 (33%)	0.92
History of CHD	8 (29%)	12 (40%)	0.36
Familiar history of CHD	5 (18%)	4 (13%)	0.63

Data are expressed as the mean value ± SD or number (%) of patients.
CHD = coronary heart disease.

drugs (except aspirin <325 mg/day) during the year preceding the study. Patients must not have had a myocardial infarction, angioplasty, severe or unstable angina pectoris, or any other cardiovascular event resulting in hospitalization during the six months preceding the study.

Laboratory determinations. Venous blood samples were collected in EDTA before the randomization visit and 6 and 12 months later in patients with FCH and four to six weeks later in patients with atherosclerotic carotid disease. The whole serum samples were stored at −80°C until analysis was performed. Lipid determinations were done using standard techniques.

Serum concentrations of sFasL were determined in duplicate with commercially available enzyme-linked immunosorbent assay kits (Diaclone Research, Besancon, France). A total of 100 μl of serum samples was assayed in parallel to known standard concentrations. Each assay was calibrated using a sFasL standard curve. The minimum detectable level of sFasL was 12 pg/ml. Intra- and interassay coefficients of variation were 4.5% and 7.1%, respectively.

Endothelial cell culture. Human microvascular endothelial cells (HMEC-1) were a gift from C. Caramelo, MD, PhD (Fundación Jiménez Díaz, Madrid, Spain). Cells were maintained in culture in MCDB-131 medium supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, 20% decomplemented fetal calf serum, 10 ng/ml epidermal growth factor, and 1 μg/ml hydrocortisone. Media were replaced every two to three days. At confluence, cells were harvested for passaging with trypsin-EDTA. Cells between passages 3 and 7 were used for the experiments. MCDB-131 and fetal calf serum were obtained from Invitrogen, Gibco (Carlsbad, California). Penicillin, streptomycin, and trypsin-EDTA were from BioWhitaker (Walkerswille, Maryland). Epidermal growth factor was from Invitrogen and hydrocortisone was from Sigma (St. Louis, Missouri). In all in vitro experiments, the ECs were preincubated for 2 h with atorvastatin, mevalonate, or isoprenoids.

Western blot analysis. Cells from different experimental conditions were collected, rinsed twice with cold phosphate-buffered saline (PBS), pelleted, and resuspended in lysis buffer containing 10% sodium dodecyl sulfate, glycerol, Tris hydrochloride, and water. Protein concentrations were calculated by the bicinchoninic acid method (Pierce, Rockford, Illinois). Equal amounts of protein were loaded into 12% acrylamide gels and electrophoresed. The resolved proteins were transferred onto polyvininylidene fluoride membranes (Immobilon, Millipore, Billerica, Massachusetts). The nonspecific sites of the membranes were blocked by incubation for 1 h at room temperature in 7.5% nonfat dry milk powder in PBS containing 0.1% Tween-20 (PBST). The membranes were incubated overnight at 4°C with rabbit anti-FasL polyclonal antibody (sc-834; Santa Cruz Biotechnology, Santa Cruz, California) or monoclonal anti-alpha-tubulin (T5168; Sigma) in PBST containing 5% nonfat dry milk. The membranes were washed with PBST and incubated for 1 h at room temperature with horseradish peroxidase–conjugated secondary antibody (Amersham, Buckinghamshire, United Kingdom) in PBST containing 5% nonfat dry milk. After a PBST wash, they were incubated for 30 min in PBST containing 400 mm NaCl, followed by detection with enhanced chemiluminescence (ECL kit, Amersham). Films were scanned on a densitometer and quantified using the ImageQuant Software (Molecular Dynamics, Sunnyvale, California).

Statistical analysis. We evaluated the effects of atorvastatin and bezafibrate in 58 patients in whom baseline, six-month, and 12-month blood samples were available. The normality of residuals was checked for each model, using the Shapiro-Wilks' *W* test. All *W* statistics for sFasL models (at baseline and 6 and 12 months) were significant ($p < 0.001$), and the hypothesis of a normal distribution was rejected. The median values and 25th and 75th percentiles at baseline and changes from baseline were computed, and rank analysis of covariance was performed to compare the effects of atorvastatin and bezafibrate from baseline. The model included the effects due to treatment and baseline sFasL as covariates. In addition, we evaluated the effects of atorvastatin or usual care in 14 patients with carotid atherosclerosis in whom baseline and four- to six-week blood samples were available.

Spearman correlation was used to make comparisons between the different parameters analyzed. The SAS version 8.2 (Cary, North Carolina) was used for analysis.

For the in vitro studies, representative data from three or four experiments are presented. To compare the group mean values, analysis of variance and the Student-Newman-Keuls test were used, as appropriate. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Atorvastatin, bezafibrate, and lipid profile in hyperlipidemic patients. Table 2 shows the changes in lipid variables

Table 2. Lipid Profile of Hyperlipidemic Patients

	Atorvastatin			Bezafibrate		
	Basal	6 Months	12 Months	Basal	6 Months	12 Months
Total cholesterol (mg/dl)	284 ± 32	195 ± 28*#	197 ± 26*#	275 ± 31	266 ± 29¶	256 ± 26§
LDL cholesterol (mg/dl)	194 ± 28	116 ± 27*#	119 ± 23*#	178 ± 24	186 ± 27	171 ± 23¶
Triglycerides (mg/dl)	266 ± 57	235 ± 67†‖	211 ± 86*	297 ± 78	184 ± 66§	202 ± 85§
HDL cholesterol (mg/dl)	37 ± 7	41 ± 6‡#	43 ± 6*‖	38 ± 8	49 ± 7§	48 ± 7§

Atorvastatin versus baseline: *$p < 0.0001$, †$p < 0.001$, ‡$p < 0.01$. Bezafibrate versus baseline: §$p < 0.0001$, ¶$p < 0.01$. Atorvastatin versus bezafibrate: #$p < 0.0001$, ‖$p < 0.01$. Data are presented as the mean value ± SD.

HDL and LDL = high- and low-density lipoprotein, respectively.

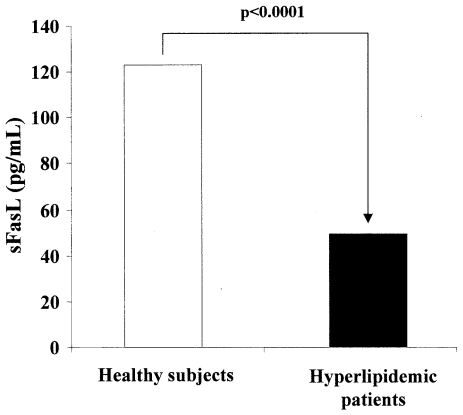

Figure 1. Effect of hyperlipidemia on solubilized Fas ligand (sFasL) levels. Representation of sFasL concentrations in healthy volunteers and hyperlipidemic patients. Data are expressed as median values.

for atorvastatin and bezafibrate at six and 12 months. At this time, atorvastatin treatment decreased total cholesterol (284 ± 32 basal vs. 195 ± 29 at 6 months and 197 ± 26 at 12 months, $p < 0.0001$), LDL cholesterol (194 ± 29 basal vs. 116 ± 27 at 6 months and 119 ± 23 at 12 months, $p < 0.0001$), and triglycerides (266 ± 57 basal vs. 235 ± 67 at 6 months, $p = 0.0004$, and 211 ± 86 at 12 months, $p < 0.0001$). On the other hand, treatment with bezafibrate led to a significant reduction of total cholesterol (256 ± 26 at 12 months, $p < 0.0001$), LDL cholesterol (171 ± 23 at 12 months, $p = 0.0014$), triglycerides (297 ± 57 basal vs. 184 ± 66 at 6 months and 202 ± 85 at 12 months, $p < 0.0001$). Atorvastatin was superior to bezafibrate in reducing total cholesterol ($p < 0.0001$) and LDL cholesterol ($p < 0.0001$), and bezafibrate was superior to atorvastatin in increasing HDL cholesterol ($p < 0.0001$ at 6 months and $p = 0.0048$ at 12 months).

Levels of sFasL in hyperlipidemic patients. To explore the association between hyperlipidemia and circulating sFasL, we measured plasma sFasL levels in 58 patients with FCH and 15 healthy volunteers. The average sFasL level in control subjects was 123 pg/ml, similar to that previously reported (17). At baseline, hyperlipidemic patients showed a significantly lower average sFasL concentration (49 pg/ml, $p < 0.0001$ vs. control subjects) (Fig. 1).

Concentrations of sFasL in atorvastatin- or bezafibrate-treated hyperlipidemic patients. We analyzed the effect of six and 12 months of treatment with atorvastatin or bezafibrate on sFasL levels in patients with combined hyperlipidemia. The basal sFasL plasma level in the atorvastatin group (n = 28) was 56 pg/ml (Fig. 2). The administration of atorvastatin significantly increased sFasL levels at six months (88 pg/ml, $p = 0.0103$ vs. basal) and 12 months (111 pg/ml, $p < 0.0001$ vs. basal), reaching normal values, without significant variations compared with healthy controls. On the other hand, the basal sFasL plasma level in the bezafibrate group (n = 30) was 46.8 pg/ml, and treatment during six months had no effect on circulating sFasL (Fig. 2). Only when the effect of 12 months of treatment was analyzed did we note that bezafibrate increases sFasL levels

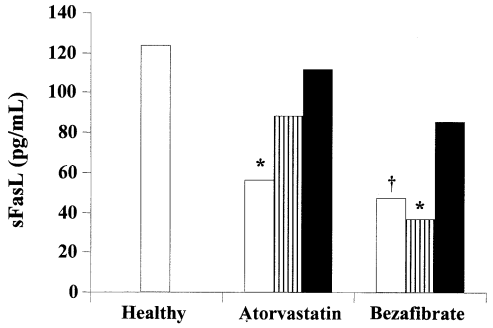

Figure 2. Effect of atorvastatin or bezafibrate treatment on circulating solubilized Fas ligand (sFasL) levels in patients with hyperlipidemia. Atorvastatin treatment normalized sFasL levels in hyperlipidemic patients at six **(striped bars)** and 12 months **(solid bars)**. **Open bars** = baseline. Data are expressed as median values. *$p < 0.0001$ and †$p < 0.0005$ compared with healthy subjects.

(85.4 pg/ml, $p = 0.046$). At baseline, sFasL plasma levels in the atorvastatin and bezafibrate groups were statistically similar. As shown in Figure 3, treatment with atorvastatin was superior to bezafibrate in normalizing sFasL levels at six ($p = 0.01$) and 12 months ($p = 0.02$). No statistical differences were found between patients treated with and those without colestiramine after 12 months of treatment.

There was no correlation between different lipid variables and sFasL plasma levels at baseline or after 6 and 12 months of treatment with atorvastatin (data not shown).

Levels of sFasL in patients with carotid atherosclerosis. To further analyze the effect of atorvastatin on sFasL levels, 14 normocholesterolemic patients without previous statin therapy but with carotid atherosclerosis were randomized to receive 80 mg/day atorvastatin (n = 7) or usual care (n = 7) during four to six weeks before they underwent carotid endarterectomy (Table 3). Treatment with atorvastatin reduced total cholesterol (186 ± 33 basal vs. 125 ± 32 after treatment, $p < 0.001$) and LDL cholesterol (123 ± 25 basal vs. 64 ± 28 after treatment, $p < 0.05$) (Table 4). In addition, we observed that patients with carotid stenosis had reduced sFasL levels (40 pg/ml), and that treatment with atorvastatin normalized sFasL concentrations, as compared with patients treated with usual care (90.7 vs. 48.3 pg/ml, respectively; $p < 0.02$) (Fig. 4). There was not any corre-

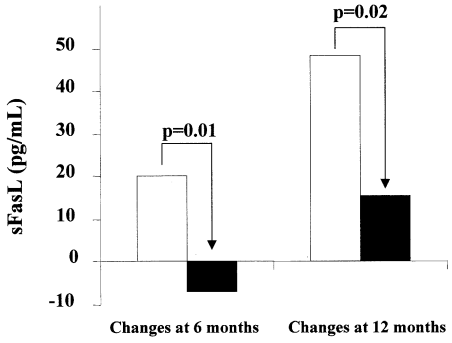

Figure 3. Changes in solubilized Fas ligand (sFasL) after treatment with atorvastatin **(open bars)** and bezafibrate **(solid bars)**. Data are expressed as the change in median value with respect to baseline.

Table 3. Characteristics of Patients With Carotid Atherosclerosis

Variable	No Treatment (n = 7)	Atorvastatin (n = 7)
Age (yrs)	68.6 ± 9	71.5 ± 6
Gender		
Men	5 (71%)	5 (71%)
Women	2 (29%)	2 (29%)
Diabetic	2 (29%)	2 (29%)
Smoker	1 (14%)	0
History of CHD	6 (86%)	6 (86%)

Data are expressed as the mean value ± SD or number (%) of patients.
CHD = coronary heart disease.

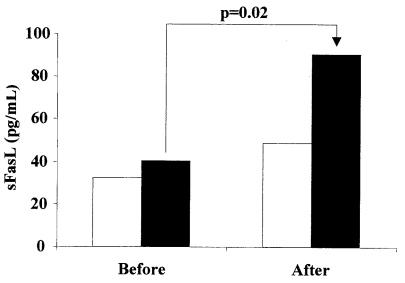

Figure 4. Effect of atorvastatin on circulating solubilized Fas ligand (sFasL) in patients with carotid atherosclerosis. Atorvastatin treatment **(open bars)** increased sFasL levels in patients with carotid atherosclerosis at four to six weeks. **Solid bars** = no treatment. Data are expressed as median values.

lation between different lipid variables and sFasL plasma levels in patients with carotid atherosclerosis. On the whole, these data indicate that atorvastatin may normalize sFasL in patients with early and late atherosclerosis.

Effect of atorvastatin on endothelial FasL. Finally, we analyzed the effect of atorvastatin on FasL expression and sFasL release in human cultured ECs. In accordance with previous studies (8), treatment with TNF-alpha decreased FasL expression in ECs (Fig. 5). To study whether atorvastatin could normalize FasL expression decreased by TNF-alpha, HMEC-1 cells were preincubated for 2 h with atorvastatin and then incubated in the presence of 25 ng/ml TNF-alpha. As shown in Figure 5A, atorvastatin prevented the diminution of FasL protein expression in cultured ECs. Furthermore, atorvastatin also prevented the diminution of sFasL levels induced by TNF-alpha in the supernatant of EC cultures (5.6 ± 2 [TNF-alpha] vs. 19.41 ± 3 [TNF-alpha plus 10 μmol/l atorvastatin] pg/ml, $p < 0.05$) (Figs. 5B and 5C). These results indicate that atorvastatin can regulate FasL expression and FasL release by ECs.

Effect of isoprenoids on endothelial FasL. Mevalonate is the metabolite that is directly synthesized by HMG-CoA reductase. We analyzed the effect of mevalonate on FasL expression. Endothelial cells were preincubated for 2 h with atorvastatin (10 μmol/l) in the presence of mevalonate (100 μmol/l) and then incubated with TNF-alpha (25 ng/ml) during 6 h. Mevalonate diminished FasL expression in the presence of atorvastatin (Fig. 6A). Moreover, mevalonate also decreased sFasL levels in the supernatant of cultured ECs (Fig. 6B).

Furthermore, we analyzed the importance of isoprenoids related to the mevalonate pathway in FasL expression, and we observed that geranylgeranyl pyrophosphate (GGPP-5, μmol/l), but not farnesyl pyrophospahte (FPP-5, μmol/l), reduced FasL expression in the presence of atorvastatin (Fig. 6A). Only GGPP was able to decrease sFasL levels in the supernatant of cultured ECs. These results indicate that GGPP, probably through geranylgeranylation of proteins, can be involved in the regulation of FasL expression.

DISCUSSION

The Fas ligand is constitutively expressed on normal ECs and may exert an atheroprotective function through its ability to induce apoptosis in mononuclear cells attempting to invade the vessel wall in the absence of normal inflammatory stimuli (6). In contrast, leukocytes express FasL only when activated. These cells are present in atherosclerotic lesions and contribute to the progression of plaque formation (18).

Membrane-bound FasL is converted to a solubilized form by a matrix metalloproteinase-like enzyme. The relevance of sFasL serum concentrations is indicated by an increment of sFasL levels observed in natural killer lymphoma (19) and rheumatic diseases (20). In addition, an increment of sFasL has been shown in patients with myocarditis (7) and in subjects with congestive heart failure (8), situations in which inflammatory cells are activated. Furthermore, Shimizu et al. (21) have recently reported that sFasL is increased in patients with myocardial infarction and unstable angina pectoris.

The Fas ligand expressed in ECs may contribute to the normal concentrations of circulating sFasL. In our study, we observed that sFasL is present in all healthy volunteers in

Table 4. Lipid Profile of Patients With Carotid Atherosclerosis

	No Treatment		Atorvastatin	
	Before	After	Before	After
Total cholesterol (mg/dl)	209 ± 64	172 ± 41	186 ± 33	125 ± 32*‡
LDL cholesterol (mg/dl)	143 ± 52	114 ± 33	123 ± 25	64 ± 28†‡
Triglycerides (mg/dl)	143 ± 52	133 ± 42	144 ± 50	100 ± 47
HDL cholesterol (mg/dl)	43 ± 13	37 ± 9	42 ± 9	41 ± 10

Before versus after: *$p < 0.01$ and †$p < 0.05$. No treatment versus atorvastatin: ‡$p < 0.05$. Data are presented as the mean value ± SD.
Abbreviations as in Table 2.

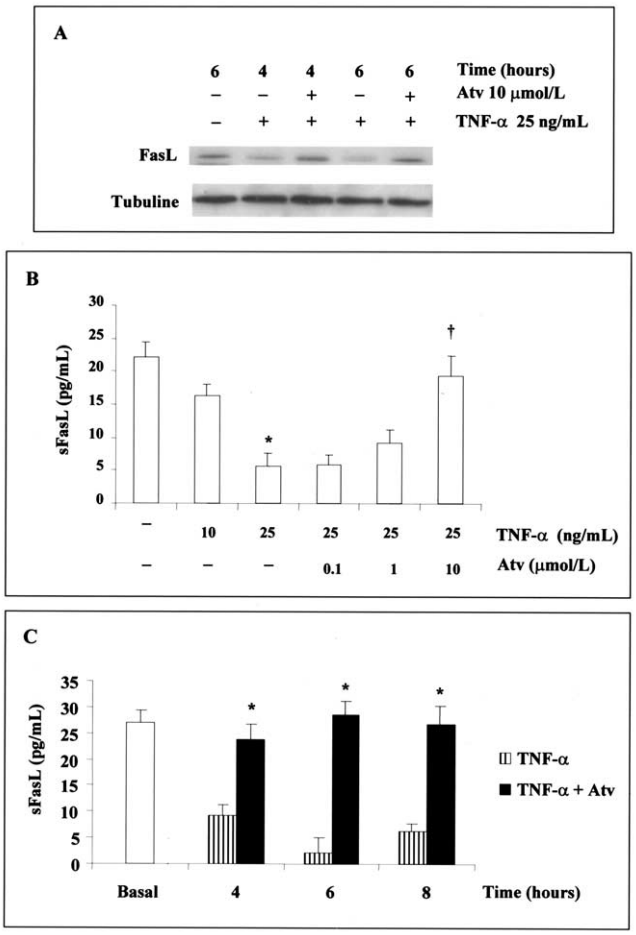

Figure 5. Effect of atorvastatin on Fas ligand (FasL) expression and solubilized Fas ligand (sFasL) released by human cultured endothelial cells (ECs). Endothelial cells were preincubated for 2 h with atorvastatin (Atv). **(A)** Western blot showing the effect of atorvastatin on FasL expression in ECs. **(B)** TNF-alpha decreased sFasL release by ECs at 6 h. Treatment with atorvastatin normalized circulating sFasL levels in a dose-dependent manner. *$p <$ 0.05 vs. baseline; †$p < 0.05$ vs. 25 ng/ml tumor necrosis factor (TNF)-alpha. **(C)** Treatment with atorvastatin (10 μmol/l) prevented the diminution of sFasL induced by TNF-alpha (*$p < 0.05$ vs. 25 ng/ml TNF-alpha).

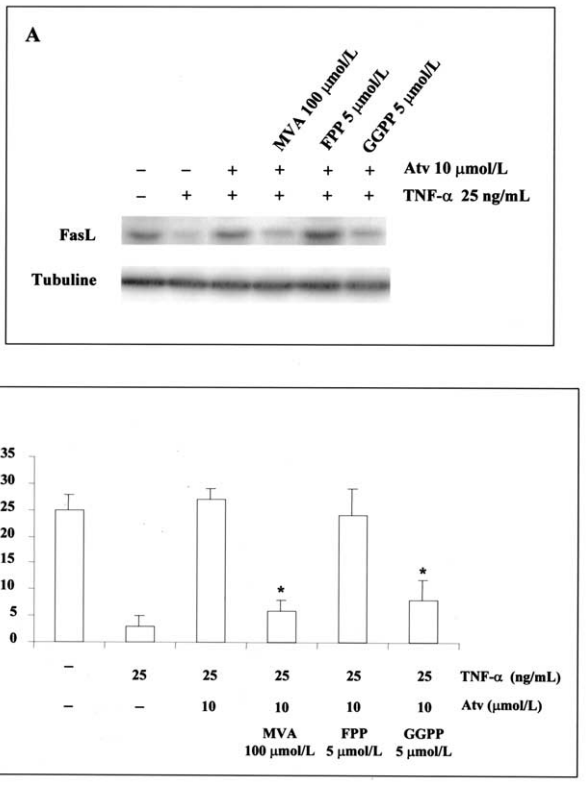

Figure 6. Effect of mevalonate (MVA) or isoprenoids on Fas ligand (FasL) expression and solubilized Fas ligand (sFasL) released by human cultured endothelial cells (ECs). Endothelial cells were preincubated for 2 h with atorvastatin (Atv), mevalonate, or isoprenoids and then 6 h with tumor necrosis factor (TNF)-alpha (25 ng/ml). **(A)** Western blot showing the effect of mevalonate, farnesyl pyrophosphate (FPP), or geranylgeranyl pyrophosphate (GGPP) on FasL expression in ECs. **(B)** Mevalonate and GGPP decreased sFasL release by ECs in the presence of atorvastatin (*$p < 0.05$ vs. atorvastatin).

concentrations considered as normal (17). However, patients with FCH have a marked decrease in sFasL levels. It is possible that the endothelial dysfunction occurring in patients with hyperlipidemia may be responsible for this finding, probably due to lesser endothelial synthesis and/or release into the blood. In contrast, increased levels of soluble cellular adhesion molecules have been found in patients with hyperlipidemia (22), and it has been attributed to the inflammation and activation of ECs (23). In this sense, it is important to remember that inflammatory stimuli, such as TNF-alpha, may upregulate adhesion molecule expression and downregulate FasL expression in ECs (6). We now show that treatment with atorvastatin prevents FasL downregulation induced by TNF-alpha in cultured ECs. Moreover, atorvastatin normalized the release of sFasL by ECs in the presence of TNF-alpha.

The lowering of cholesterol plasma levels by diet or drugs downregulates adhesion molecule expression and reduces the density of inflammatory cells in atherosclerotic plaques (24). The present measurements show that the changes in circulating sFasL reflect analogous effects of statins on ECs. This suggests that statins may inhibit the recruitment of leukocytes associated with hypercholesterolemia by increasing endothelial FasL expression. Our results also indicate that the normalization of sFasL levels is not simply due to the reversal of hypercholesterolemia, because we observed an analogous sFasL increase in patients whose plasma cholesterol fell within the normal range. However, even in these patients, the increase in sFasL was accompanied by a highly significant further reduction of cholesterol levels. Moreover, although the cholesterol-lowering effect of atorvastatin was far more powerful than that of bezafibrate, prolonged fibrate treatment also raised sFasL. Thus, future studies will have to establish whether the mechanism responsible for the increase in sFasL is truly independent of cholesterol lowering.

Several cholesterol-independent effects of statins in vitro have been reported, such as the statin-induced upregulation of endothelial nitric oxide mediated by a decrease in GGPP levels, an isoprenoid intermediate of the cholesterol synthesis pathway, and inhibition of the geranylgeranylation of Rho proteins (25). In addition, other cholesterol-independent ef-

fects of statins may also be mediated by the inhibition of Rho guanosine triphosphatase, such as inhibition of the cell-cycle progression of vascular smooth muscle cells or the release of tissue plasminogen activator (26,27). In this sense, we also noted that FasL expression is controlled by GGPP in human ECs, indicating that isoprenoids can contribute to its regulation.

The normalization of sFasL by atorvastatin may contribute to a decrease in the influx of inflammatory cells to the vessel wall and prevent the progression of plaque formation. In this sense, FasL-deficient mice display enhanced leukocyte infiltration and intima hyperplasia (28), and FasL gene transfer to the vessel wall can effectively inhibit the vessel lesion formation (29).

Conclusions. The present study shows, for the first time, that patients with FCH have low circulating sFasL levels, and that treatment with atorvastatin normalizes them. Moreover, it provides an additional explanation by which hyperlipidemia may contribute to the development of atherosclerotic lesions: high lipids might alter the regulation of inflammatory cells extravasation. In addition, atorvastatin also normalized sFasL levels in normocholesterolemic patients with carotid stenosis. Furthermore, atorvastatin prevented FasL downregulation and sFasL release in cytokine-stimulated ECs. However, the mechanisms by which hyperlipidemia or carotid atherosclerosis reduce sFasL levels and atorvastatin normalizes them remain to be elucidated.

Further studies are needed to assess whether the decrease in sFasL levels constitutes a marker of endothelial dysfunction. Experiments to explore this possibility are currently underway in our laboratory in patients with various diseases.

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